Isolation and Characterization of Bovine Mammary Calmodulin

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ABSTRACT

Bovine mammary gland calmodulin, purified by conventional fractionation procedures, was compared with similarly purified bovine brain calmodulin. Affinity chromatography on W-7 agarose of the crude fractions from mammary gland and brain yielded pure proteins containing one trimethyllysine residue per 16,800 daltons with essentially identical amino acid compositions. Kinetic parameters of these two proteins with respect to their ability to activate phosphodiesterase were determined. The constants for half maximum activation were .39 and .44 n*M* for bovine brain and bovine mammary gland calmodulins, respectively; both proteins gave similar maximum velocities. Based on the amino acid composition and kinetic data, it is concluded that the two proteins are essentially identical.

INTRODUCTION

Calmodulin (CaM) is a low molecular weight (bovine brain CaM = 16,800 daltons) acidic protein ubiquitous in eukaryotic cells of both animals and plants. It binds 4 Ca^{2+} per molecule and is the well-known activator of cyclic nucleotide phosphodiesterase (PDE) (7). Calmodulin has multiple cell functions, including regulation of cell division and activation of numerous enzymes (23), including NAD^+ kinase in plant systems (16). An additional feature of the protein is the presence of the unusual amino acid, trimethyllysine (TML), lysine being methylated posttranslationally). To date, all isolated animal CaM contain TML, but CaM isolated from *Chlamydomonas*, a photosynthetic alga, contains no TML and yet stimulates PDE (10). Many plant CaM contain cysteine, whereas no animal CaM has been observed to contain that amino acid.

Calmodulin occurs in bovine mammary tissue and has been partially characterized by Riss and Baumrucker (17, 18). Based upon the activation of PDE and the molecular weight determination by SDS-PAGE, it was concluded that mammary tissue contains CaM. According to Riss and Baumrucker (17) and Thompson et al. (20, 21), bovine milk, however, does not contain CaM. In this paper, we report the isolation and characterization of CaM from bovine mammary tissue and directly compare the properties of mammary CaM with similarly isolated bovine brain CaM (22). In particular, we compare the amino acid composition of these two proteins with the amino acid sequence analyses of Watterson et al. (24) to determine if bovine mammary CaM and bovine brain CaM are identical. In addition, we compare kinetic parameters between the two proteins in their ability to activate PDE.

MATERIALS AND METHODS

Sources of Tissue

Fresh calf brains and hearts were obtained from a local slaughter house and stored at −80°C unless used immediately. Mammary gland was obtained from a slaughtered, lactating Holstein cow; the gland was to cut in 300-g pieces and frozen at −30°C until used.

Preparation of Cyclic Adenosine Monophosphate Phosphodiesterase

Activator (CaM)-deficient PDE was prepared from calf hearts according to the procedure of Ho et al. (12) modified by the use of DE-52 cellulose rather than regular DEAE-cellulose and by elution of the enzyme using an EDTA containing buffer. Highly active PDE, eluted from DE-52, was frozen and stored in 1-ml aliquots at −80°C until used.

Received December 8, 1986.
Accepted March 19, 1987.

Preparation of Crude Bovine Brain Calmodulin and Mammary Calmodulin

Calmodulin in Calf Brain. Two calf brains (about 700 g) were thawed overnight at 6°C, diced, weighed, and homogenized at 4°C in a Waring blender[1] with short spurts at low speeds in two volumes of a buffer at pH 8.0 consisting of 1 m*M* EDTA, 10 m*M* mercaptoethanol, and 20 m*M* Tris. The homogenized tissue was filtered through doubled cheesecloth and centrifuged at 16,000 rpm in a Sorvall centrifuge for 35 min at 4°C. The supernatant was brought to 50% of saturation with ammonium sulfate, stirred for 30 min, and the precipitate was removed by centrifugation at 16,000 rpm for 35 min. After addition of solid ammonium sulfate to make the supernatant 70% of saturation, the fraction was centrifuged as before. The pellet was dissolved in a minimum of homogenization buffer, heated at 85°C for 5 min, immediately cooled to 2 to 4°C in an ice bath, and centrifuged as before. The supernatant was dialyzed overnight against buffer A (20 m*M* Tris and 100 μM $CaCl_2$, pH 7.5) in preparation for affinity chromatography. Following dialysis, any insoluble material was removed by centrifugation.

Mammary Calmodulin. Calmodulin from mammary tissue (.5 × 1 kg) was prepared similarly to brain CaM, but because of the viscous nature of the homogenate either the volume of buffer was doubled or the filtration step was eliminated.

Purification of Calmodulin by W-7 Agarose Chromatography

Final purification of bovine and mammary CaM was achieved by calcium-dependent affinity chromatography, the CaM antagonist, W-7, coupled to agarose. As discussed by Endo et al. (9), the antagonist may also be coupled to Sepharose. The principle of the separation of CaM from other proteins by affinity chromatography (W-7 agarose) is that CaM, in the presence of Ca^{2+}, binds strongly to the antagonist. Although other Ca^{2+} modulated proteins may also bind to the immobilized antagonist, they do so with much less affinity.

Fifteen milliliters of W-7 [N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide] agarose (Sigma) was poured into a 1 × 20 cm glass column plugged with glass wool and rinsed exhaustively with buffer A (pH 7.5, 20 m*M* Tris, 5 m*M* $MgCl_2$, 5 m*M* NaCl) to remove excess W-7. Dialyzed, crude CaM (100 to 150 ml) was applied to the column at a flow rate 2 ml/m, and the column was washed with buffer A until absorbance at 280 nm reached zero. Elution was continued using buffer A containing .3 *M* NaCl. Calmodulin eluted with pH 7.5 buffer consisting of 20 m*M* Tris, 5 m*M* $MgCl_2$, 2 m*M* EGTA, and .5 *M* NaCl. Following elution, the solution was adjusted to 10 to 20 m*M* $CaCl_2$ and dialyzed for at least 5 d against twice daily changes of deionized water to remove salts. The dialyzed protein solution was adjusted to pH 7.5 with .1 N NaOH, lyophilized, and stored (desiccated) at −20°C.

Amino Acid Analyses

Twenty-four-hour acid hydrolysates (6 *M* HCl) of CaM were analyzed in triplicate using a Beckman 119 CL amino acid analyzer. [^{6}N] Trimethyllysine was identified by comparison of retention time with a standard.

Protein Analyses

Protein concentration (microassay procedure) was determined by the Coomassie method of Bradford (2) using the reagent supplied by Bio Rad and their protein standard.

Phosphodiesterase Assay

It has been demonstrated by Cheung (7) that activator-dependent cyclic AMP (cAMP) PDE requires CaM as the activator. The percent activation of PDE was determined by comparison of the rate of hydrolysis of cAMP with CaM added to those containing no CaM. The rate of hydrolysis by fully activated PDE was arbitrarily assigned the value of 100%. The assay was performed as described by Butcher (5) except that assays were run at room temperature and $CaCl_2$ (18 μM) and CaM were present. Further, each assay tube was centrifuged to remove turbidity immediately before reading absorbance at 650 nm.

[1] Reference to brand or firm name does not constitute endorsement by the US Department of Agriculture over others of a similar nature not mentioned.

Polyacrylamide Gel Electrophoresis

The PAGE and SDS-PAGE were run as described by Thompson et al. (21). Molecular weight standards for SDS-PAGE included α-lactalbumin (14 kdalton), β-lactoglobulin (18 kdalton), ovalbumin (43 kdalton), and bovine serum albumin (67 kdalton).

RESULTS AND DISCUSSION

Purification of Calmodulin

The procedure described works well for the isolation bovine brain and mammary CaM. The purity of the preparations by PAGE and SDS-PAGE is demonstrated (Figures 1 and 2). There is little contamination with other proteins, notably S100, which would have migrated with a molecular weight of approximately 11 to 12 kdalton. Molecular weights of both CaM were identical by SDS-PAGE, i.e., 17,000 daltons.

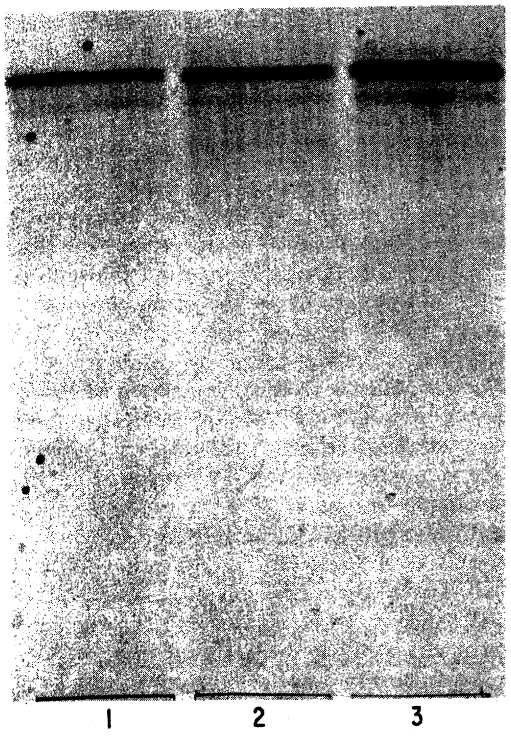

Figure 1. Alkaline PAGE of commercial calf brain calmodulin (CaM), bovine mammary CaM (this study), and calf brain CaM (this study), lanes 1, 2, and 3. Protein concentration was approximately 20 μg/slot in lanes 1 and 2, and 40 μg/slot in lane 3.

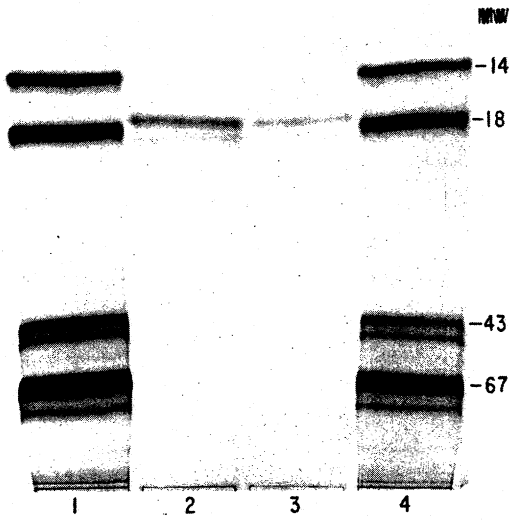

Figure 2. The SDS-PAGE of molecular weight standards (lanes 1 and 4). Lanes 2 and 3 represent calf brain calmodulin (CaM) and bovine mammary CaM, respectively. Protein concentration was approximately 70 μg/slot in lanes 1 and 4, 20 μg/slot in lane 2, and 15 μg/slot in lane 3. MW = Molecular weight.

With this procedure we recovered approximately 30 mg CaM/kg mammary tissue, which is similar to the 24 to 51 mg CaM/500 g tissue reported by Riss and Baumrucker (17), who used PDE assay methods as a means of determining yield. Klee (13) reported a yield of 162 mg CaM/kg of pig brain from acetone powders, whereas Gopalakrishna and Anderson (11) obtained 160 to 180 mg CaM/kg bovine brain when purification was by hydrophobic interaction chromatography (see next paragraph). In this study, our yields for calf brain CaM approximately 100 mg/kg of brain tissue, were lower than those just reported (11).

Alternatively, we have employed two methods to purify bovine and mammary CaM in addition to that described. One method, that of Schreiber et al. (19) for the purification of CaM and CaM fragments from human brain, simplifies fractionation and purification by elimination of salt fractionation procedures. Instead, chromatography on DE-52 cellulose and on Sephadex G-75 purifies the protein. The second method involves the purification of CaM by hydrophobic interaction chromatography (HIC) on phenyl Sepharose (11). We have

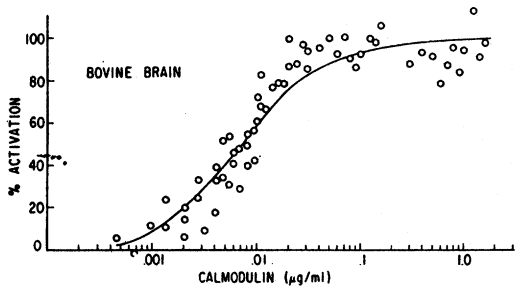

Figure 3. Activation of heart cyclic AMP phosphodiesterase by bovine brain calmodulin (this study, see text).

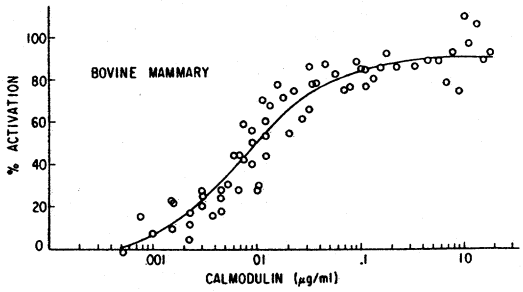

Figure 4. Activation of heart cyclic AMP phosphodiesterase by bovine mammary calmodulin (this study, see text).

observed that phenyl Sepharose is less "poisoned" during chromatography than is W-7 agarose and may be reused more frequently than the latter. In addition, the column capacity is significantly greater with HIC (11). However, the purity of the proteins, obtained by all methods herein mentioned, is essentially identical. Therefore, we have reported data only on CaM obtained by our method.

Kinetic Parameters

Mammary CaM was compared with calf brain CaM for ability to activate PDE (Figures 3 and 4). The constant for half maximal activation, K_a, for calf brain CaM and bovine mammary CaM were .39 ± .04 and .44 ± .04 n*M*, respectively, whereas the stimulated maximum velocity for each protein was 100 ± 2.5 and 91 ± 2.2%, respectively. The K_a for brain CaM are comparable to literature values, which range from .20 to 1.2 n*M* (6). Recalculation of the kinetic data given by Riss and Baumrucker (17) to zero basal activity gives a K_a of .59 n*M*.

Animo Acid Analysis

Table 1 shows the amino acid analyses of calf brain and bovine mammary CaM (this study) compared with the amino acid sequence analysis of bovine brain CaM (24). Typically for CaM, both proteins contain 1 TML per molecule and no tryptophan or cysteine residues. Compared with the sequence analysis, our data are slightly higher for serine, proline, and lysine but lower for threonine; however, these data reflect only one-time hydrolysis (24 h). Analysis by the Cornish-Bowden (8) equation (S△n) shows the two isolated CaM to be nearly identical (Table 2). The data also suggest that bovine mammary CaM may be slightly purer than similarly isolated calf brain CaM. The lower K_a for calf brain CaM, however, does not support this suggestion.

Reported Biological Roles of Calmodulin

The main objective of this study was to compare the properties and composition of bovine mammary CaM with that of calf brain CaM and to use these proteins as reference standards for research on other biological systems. Although we have not attempted to discover roles for CaM in mammary functions, it is important to briefly mention the reported roles of this protein in the gland. Brooks and Landt (3, 4) reported the presence of CaM-dependent κ-casein kinase in mammary tissue, which required a CaM concentration of 204 n*M* in the presence of Ca^{2+} to achieve one-half maximal stimulation. This value is somewhat high compared with the .39 n*M* found for PDE in this study but is within the potential concentration of CaM, estimated from recovery to be at least 1.7 μ*M* (30 mg/kg) in bovine mammary gland. On mediation of hormone action (14), Merritt et al. (15) suggested that CaM might play a role in prolactin secretion.

Bolander (1) proposed that the Ca^{2+} · CaM system is involved in prolactin-induced differentiation of mammary gland explants; he pointed out, however, that CaM is not the mediator of prolactin action. Riss et al. (18) compared levels of CaM in rat tissue during the course of lactation and observed a 2.5-fold

TABLE 1. Amino acid analyses of calf brain and bovine mammary calmodulins (this study) as compared with the sequence analysis of bovine brain calmodulin (24).

Amino acid	Brain		Mammary gland		Sequence (brain)
	(μmol)[1]	σ	(μmol)[1]	σ	(μmol)
Aspartic acid	20.70	.47	22.15	.62	23
Threonine	10.40	.10	11.38	.45	12
Serine	5.71	.24	5.08	.05	4
Glutamic acid	25.97	.69	27.50	.31	27
Proline	3.46	.19	2.95	.05	2
Glycine	11.00	Fixed	11.00	Fixed	11
Alanine	10.81	.45	11.08	.15	11
½Cystine[2]	...	...	...	...	0
Valine	6.79	.72	6.73	.47	7
Methionine	7.22	.30	7.79	.17	9
Isoleucine	6.93	.16	8.16	.48	8
Leucine	8.91	.20	10.30	.27	9
Tyrosine	2.31	.10	2.39	.06	2
Phenylalanine	7.15	.13	7.90	.16	8
Lysine	8.36	.12	8.10	.19	7
Histidine	1.45	.16	1.26	.06	1
Tryptophan	...	...	...	...	0
Arginine	6.25	.23	6.30	.30	6
TML[3]	.94	.04	.95	.08	1

[1] Average (± σ) for three analyses; hydrolysis time = 24 h.

[2] Not determined.

[3] TML = Trimethyllysine.

increase near the time of parturition, which plateaud during lactation and dropped only at involution.

CONCLUSIONS

Kinetic data and amino acid analyses indicate that mammary CaM is nearly identical to calf brain CaM. This is in accord with the high degree of homology found for CaM both across species and within tissues of single species (6). Verification of complete identity could only be obtained by amino acid sequence analysis or monoclonal antibodies to the proteins. Mammary tissue is thus a relatively good source of CaM, and the protein is interchangeable in the phosphodiesterase assay system with bovine brain CaM.

We observed other Ca^{2+}-binding proteins in mammary tissue homogenates, including S100, an 11,500 dalton protein whose molecular target is fructose-1,6-bisphosphate aldolase (25). Other important Ca^{2+}-binding proteins will likely be discovered and their biological roles elucidated.

TABLE 2. Comparison of amino acid compositions of calmodulins (CaM) by the SΔn method of Cornish-Bowden.[1]

CaM	Sequence CaM	Mammary CaM
Mammary	4.0	
Brain	8.8	2.4

[1] Strong correlation exists for a protein of 18,000 daltons when SΔn ≤61.6. Values well below this number mean the proteins are identical within the error of the analyses.

ACKNOWLEDGMENT

We are grateful to Harold J. Dower for performing amino acid analyses.

REFERENCES

1 Bolander, F. F., Jr. 1985. Possible roles of calcium and calmodulin in mammary gland differentiation *in vitro*. J. Endocrinol. 104:29.

2 Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72:248.
3 Brooks, C. L., and M. Landt. 1984. Calcium-ion and calmodulin-dependent κ-casein kinase in rat mammary acini. Biochem. J. 224:195.
4 Brooks, C. L., and M. Landt. 1985. Calmodulin-dependent protein kinase in acini from lactating rat mammary tissue: subcellular localization, characterization and solubilization. Arch. Biochem. Biophys. 240:663.
5 Butcher, R. W. 1974. Cyclic 3′, 5′-nucleotide phosphodiesterase from bovine heart. Pages 218–223 *in* Methods in enzymology. Vol. 38. S. P. Colowick and N. O. Kaplan, ed. Academic Press, New York, NY.
6 Chau, V., C. Y. Huang, P. B. Chock, J. H. Wang, and R. K. Sharma. 1982. Kinetic studies of the activation of cyclic nucleotide phosphodiesterase by calcium and calmodulin. Pages 199–217 *in* Calmodulin and intracellular calcium receptors. S. Kakiuchi, H. Hidaka, and A. R. Means, ed. Plenum Press, New York, NY.
7 Cheung, W. Y. 1967. Cyclic 3′, 5′-nucleotide phosphodiesterase. Pronounced stimulation by snake venom. Biochem. Biophys. Res. Commun. 29:478.
8 Cornish-Bowden, A. 1983. Relating proteins by amino acid composition. Page 60 *in* Methods in enzymology. Vol. 91. S. P. Colowick and N. O. Kaplan, ed. Academic Press, New York, NY.
9 Endo, T., T. Tanaka, T. Isobe, H. Kasai, T. Okuyama, and H. Hidaka 1981 Calcium-dependent affinity chromatography of S100 and calmodulin on Calcium Antagonist-coupled sepharose. J. Biol. Chem 256:12485.
10 Gitelman, S. E., and G. B. Whitman. 1980. Purification of calmodulin from *Chlamydomonas:* calmodulin occurs in cell bodies and flagella. J. Cell Biol. 87:764.
11 Gopalarkrishna, R., and W. B. Anderson. 1982. Ca^{2+}-induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-Sepharose affinity chromatography. Biochem. Biophys. Res. Commun. 104:830.
12 Ho, H. C., T. S. Teo, R. Desai, and J. H. Wang. 1976. Catalytic and regulatory properties of two forms of bovine heart cyclic nucleotide phosphodiesterase. Biochem. Biophys. Acta 429:461.
13 Klee, C. B. 1977. Conformational transition accompanying the binding of Ca^{2+} to the protein activator of 3′, 5′-cyclic adenosine monophosphate phosphodiesterase. Biochemistry 16:1017.
14 Means, A. R., L. Lagace, V. Guerriero, Jr., and J. G. Chafouleas. 1982. Calmodulin as a mediator of hormone action and cell regulation. J. Cell. Biochem. 20:317.
15 Merritt, J. E., S. MacNeil, S. Tomlinson, and B. L. Brown. 1983. The relationship between prolactin secretion and calmodulin activity. J. Endocrinol. 98:423.
16 Muto, S., S. Miyachi, H. Usuda, G. E. Edwards, and J. A. Bassham. 1981. Light-induced conversion of nicotinamide adenine dinucleotide to nicotinamide adenine dinucleotide phosphate in higher plants. Plant Physiol. 68:324.
17 Riss, T. L., and C. R. Baumrucker. 1982. Calmodulin purification and quantitation from bovine mammary tissue. J. Dairy Sci. 65:1722.
18 Riss, T. L., P. J. Bechtel, and C. R. Baumrucker. 1984. Calmodulin content of rat mammary tissue and isolated cells during pregnancy and lactation. Biochem. J. 219:927.
19 Schreiber, W. E., T. Sasagawa, K. Titani, R. D. Wade, D. Malencik, and E. H. Fischer. 1981. Human brain calmodulin: isolation, characterization, and sequence of a half-molecule fragment. Biochemistry 20:5239.
20 Thompson, M. P., and D. P. Brower. 1985. Calcium binding proteins in bovine milk. J. Dairy Sci. 68(Suppl. 1):59. (Abstr.)
21 Thompson, M. P., D. P. Brower, and H. M. Farrell, Jr. 1987. Absence of detectable calmodulin in cow's milk by a modified gel electrophoresis method. J. Dairy Sci. 70:1134.
22 Thompson, M. P., G. J. Piazza, D. P. Brower, E. W. Bingham, and H. M. Farrell, Jr. 1986. Isolation and properties of bovine mammary calmodulin. Fed. Proc. 45:1238.
23 Watterson, D. M., W. G. Harrelson, Jr., P. M. Keller, F. Sharief, and T. C. Vanaman. 1976. Structural similarities between the Ca^{2+}-dependent regulatory proteins of 3′:5′-cyclic nucleotide phosphodiesterase and actomyosin ATPase. J. Biol. Chem. 251:4501.
24 Watterson, D. M., F. Sharief, and T. C. Vanaman. 1980. The complete amino acid sequence of the Ca^{2+}-dependent modulator protein (calmodulin) of bovine brain. J. Biol. Chem. 255:962.
25 Zimmer, D. B., and L. J. Van Eldik. 1986. Identification of a molecular target for the calcium-modulated protein S100. J. Biol. Chem. 261: 11424.